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**PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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INVENTOR(S)					
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<input checked="" type="checkbox"/> Additional Inventors are being named on the 1 separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
MRBs as MODIFIERS OF THE RB PATHWAY AND METHODS OF USE					
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No.					
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Respectfully submitted,  
SIGNATURE

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8/7/2002

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47,937

Docket Number:

EX02-109P

**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231

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**PROVISIONAL APPLICATION COVER SHEET**  
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Docket Number		EX02-109P
<b>INVENTOR(S)/APPLICANT(S)</b>		
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Number 1 of 1

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**MRBs as MODIFIERS OF THE RB PATHWAY AND METHODS OF USE****BACKGROUND OF THE INVENTION**

Retinoblastoma, a pediatric eye tumor, has served as an important model for the heritable predisposition to cancer. The primary mechanism in the development of retinoblastoma is loss or inactivation of both alleles of this gene (Murphree, A. L. and Benedict, W. F. (1984) *Science* 223: 1028-1033). The high incidence of second primary tumors among patients who inherit one retinoblastoma gene suggests that this cancer gene plays a key role in the etiology of several other primary malignancies.

The retinoblastoma protein, RB, functions as a tumor suppressor by controlling progression through the cell cycle which is achieved by sequestering a variety of nuclear proteins involved in cellular growth. Thus, it acts as a signal transducer connecting the cell cycle clock with the transcriptional machinery (Weinberg, R. A. (1995) *Cell* 81: 323-330). RB regulates cell proliferation by restricting cell cycle progression at a specific point in G1, by interaction with the E2F family of transcription factors to arrest cells in G1 (Goodrich, D. W. et al. (1991) *Cell* 67: 293-302; Zhang, H. S. et al. (1999) *Cell* 97: 53-61).

RB function is regulated primarily by its phosphorylation state, which is determined by the complex interaction of multiple kinases and their inhibitors that together form the 'Rb pathway' (DeCaprio, J. A. et al (1989) *Cell* 58: 1085-1095; Buchkovich, K. et al (1989) *Cell* 58: 1097-1105; Chen, P.-L. et al. (1989) *Cell* 58: 1193-1198). This pathway has been found to be functionally inactivated in almost all types of cancer.

RB sequence is conserved in evolution, and exists in mouse (Bernards R et al (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:6474-6478), rat (Roy NK et al. (1993) *Nucleic Acids Res.* 21:170-170), *Drosophila* (Du W et al (1996) *Genes Dev* 10:1206-18), and *C. elegans* (The *C. elegans* Sequencing Consortium (1998) *Science* 282:2012-2018).

PSMC3 (Proteasome (prosome macropain) 26S ATPase subunit 3 (Tat binding protein)) is a protein that interacts with the human immunodeficiency virus Tat transactivator (Nelbock, P. et al (1990) *Science* 248: 1650-1653). PSMC3 is also part of

both PA700 and PA700-dependent regulatory complexes (DeMartino, G. N. et al (1996) J. Biol. Chem. 271: 3112-3118).

Ribosomal protein S6 (RPS6) is the major substrate of protein kinases in eukaryotic ribosomes. RPS6 is a structural component of the cytosolic small (40S) ribosomal subunit, and functions in RNA binding and protein biosynthesis.

RPS6 is upregulated in colon/large intestine adenocarcinoma and carcinoma primary tumors, and is expressed in Hepatocarcinoma primary tumor, mammary gland/breast adenocarcinoma cell lines, and stomach carcinoma primary tumors (Pogue-Geile, K. et al (1991) Mol Cell Biol 11:3842-9; Barnard, G. F. et al (1993) Cancer Res 53:4048-52; Penner, C. G. ET AL (1991) Mol Cell Biochem 108:57-66).

The ability to manipulate the genomes of model organisms such as *Drosophila* provides a powerful means to analyze biochemical processes that, due to significant evolutionary conservation, have direct relevance to more complex vertebrate organisms. Due to a high level of gene and pathway conservation, the strong similarity of cellular processes, and the functional conservation of genes between these model organisms and mammals, identification of the involvement of novel genes in particular pathways and their functions in such model organisms can directly contribute to the understanding of the correlative pathways and methods of modulating them in mammals (see, for example, Mechler BM et al., 1985 EMBO J 4:1551-1557; Gateff E. 1982 Adv. Cancer Res. 37: 33-74; Watson KL., et al., 1994 J Cell Sci. 18: 19-33; Miklos GL, and Rubin GM. 1996 Cell 86:521-529; Wassarman DA, et al., 1995 Curr Opin Gen Dev 5: 44-50; and Booth DR. 1999 Cancer Metastasis Rev. 18: 261-284). For example, a genetic screen can be carried out in an invertebrate model organism or cell having underexpression (e.g. knockout) or overexpression of a gene (referred to as a "genetic entry point") that yields a visible phenotype, such as altered cell growth. Additional genes are mutated in a random or targeted manner. When a gene mutation changes the original phenotype caused by the mutation in the genetic entry point, the gene is identified as a "modifier" involved in the same or overlapping pathway as the genetic entry point. When inactivation of either gene is not lethal, but inactivation of both genes results in reduced viability or death of the cell, tissue, or organism, the interaction is defined as "synthetic lethal"

(Bender, A and Pringle J, (1991) Mol Cell Biol, 11:1295-1305; Hartman J et al, (2001) Science 291:1001-1004; Published application WO0151604). In a synthetic lethal interaction, the modifier may also be identified as an "interactor". When the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as RB, modifier genes can be identified that may be attractive candidate targets for novel therapeutics.

All references cited herein, including sequence information in referenced Genbank identifier numbers and website references, are incorporated herein in their entireties.

### SUMMARY OF THE INVENTION

We have discovered genes that modify the RB pathway in *Drosophila* cells, and identified their human orthologs, hereinafter referred to as MRB. The invention provides methods for utilizing these RB modifier genes and polypeptides to identify MRB-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired RB function and/or MRB function. Preferred MRB-modulating agents specifically bind to MRB polypeptides and restore RB function. Other preferred MRB-modulating agents are nucleic acid modulators such as antisense oligomers and RNAi that repress MRB gene expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

MRB modulating agents may be evaluated by any convenient *in vitro* or *in vivo* assay for molecular interaction with an MRB polypeptide or nucleic acid. In one embodiment, candidate MRB modulating agents are tested with an assay system comprising a MRB polypeptide or nucleic acid. Agents that produce a change in the activity of the assay system relative to controls are identified as candidate RB modulating agents. The assay system may be cell-based or cell-free. MRB-modulating agents include MRB related proteins (e.g. dominant negative mutants, and biotherapeutics); MRB-specific antibodies; MRB-specific antisense oligomers and other nucleic acid modulators; and chemical agents that specifically bind to or interact with MRB or compete with MRB binding partner (e.g. by binding to an MRB

binding partner). In one specific embodiment, a small molecule modulator is identified using a binding assay. In specific embodiments, the screening assay system is selected from an apoptosis assay, a cell proliferation assay, an angiogenesis assay, and a hypoxic induction assay.

In another embodiment, candidate RB pathway modulating agents are further tested using a second assay system that detects changes in the RB pathway, such as angiogenic, apoptotic, or cell proliferation changes produced by the originally identified candidate agent or an agent derived from the original agent. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating the RB pathway, such as an angiogenic, apoptotic, or cell proliferation disorder (e.g. cancer).

The invention further provides methods for modulating the MRB function and/or the RB pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a MRB polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to a mammalian animal predetermined to have a pathology associated the RB pathway.

### DETAILED DESCRIPTION OF THE INVENTION

The Rb co-RNAi synthetic lethal screen was designed to identify modifier genes that are synthetic lethal with the *Drosophila* Rbf gene (Du W et al (1996) *supra*), a *Drosophila* homolog of the human retinoblastoma (RB) gene. In addition to identifying modifier genes with synthetic lethal interactions with Rbf, this screen identified modifier genes that, when inactivated, preferentially reduced the viability of Rbf-deficient cells relative to normal cells. Vertebrate orthologs of these modifiers, and preferably the human orthologs, MRB (Modifier of RB) genes (i.e., nucleic acids and polypeptides) are attractive drug targets for the treatment of pathologies associated with a defective RB signaling pathway, such as cancer. Table 1 lists the modifiers and their orthologs.

In vitro and in vivo methods of assessing MRB function are provided herein.

Modulation of the MRB or their respective binding partners is useful for

understanding the association of the RB pathway and its members in normal and disease conditions and for developing diagnostics and therapeutic modalities for RB related pathologies. MRB-modulating agents that act by inhibiting or enhancing MRB expression, directly or indirectly, for example, by affecting an MRB function such as enzymatic (e.g., catalytic) or binding activity, can be identified using methods provided herein. MRB modulating agents are useful in diagnosis, therapy and pharmaceutical development.

### **Nucleic acids and polypeptides of the invention**

Sequences related to MRB nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) number), shown in Table 1 and in the appended sequence listing .

Preferred MRBs are ATPases and RNA binding proteins. The term "MRB polypeptide" refers to a full-length MRB protein or a functionally active fragment or derivative thereof. A "functionally active" MRB fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type MRB protein, such as antigenic or immunogenic activity, enzymatic activity, ability to bind natural cellular substrates, etc. The functional activity of MRB proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey) and as further discussed below. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of an MRB, such as a binding domain. Protein domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2). Methods for obtaining MRB polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of an MRB. In further preferred embodiments, the fragment comprises the entire functionally active domain.



The term "MRB nucleic acid" refers to a DNA or RNA molecule that encodes a MRB polypeptide. Preferably, the MRB polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with MRB. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD *et al.*, 1994, Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *Drosophila*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the

program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, *Advances in Applied Mathematics* 2:482-489; database: European Bioinformatics Institute; Smith and Waterman, 1981, *J. of Molec.Biol.*, 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" ([www.psc.edu](http://www.psc.edu)) and references cited therein.; W.R. Pearson, 1991, *Genomics* 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff (Dayhoff: *Atlas of Protein Sequences and Structure*, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 *Nucl. Acids Res.* 14(6):6745-6763). The Smith-Waterman algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated, the "Match" value reflects "sequence identity."

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of an MRB. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of an MRB under stringent hybridization conditions that comprise: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 µg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.2X SSC and 0.1% SDS (sodium dodecyl sulfate).

In other embodiments, moderately stringent hybridization conditions are used that comprise: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Alternatively, low stringency conditions can be used that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

**Isolation, Production, Expression, and Mis-expression of MRB Nucleic Acids and Polypeptides**

MRB nucleic acids and polypeptides, useful for identifying and testing agents that modulate MRB function and for other applications related to the involvement of MRB in the RB pathway. MRB nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods. For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of an MRB protein for assays used to assess MRB function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (*e.g.*, Higgins SJ and Hames BD (eds.) *Protein Expression: A Practical Approach*, Oxford University Press Inc., New York 1999; Stanbury PF et al., *Principles of Fermentation Technology*, 2<sup>nd</sup> edition, Elsevier Science, New York, 1995; Doonan S (ed.) *Protein Purification Protocols*, Humana Press, New Jersey, 1996; Coligan JE et al, *Current Protocols in Protein Science* (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant MRB is expressed in a cell line known to have defective RB function (*e.g.* SAOS-2 osteoblasts, BT549 breast cancer cells, and C33A cervical cancer cells, among others, available from American Type Culture Collection (ATCC), Manassas, VA). The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

The nucleotide sequence encoding an MRB polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals,

including promoter/enhancer element, can derive from the native MRB gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. A host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

To detect expression of the MRB gene product, the expression vector can comprise a promoter operably linked to an MRB gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the MRB gene product based on the physical or functional properties of the MRB protein in *in vitro* assay systems (*e.g.* immunoassays).

The MRB protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (*i.e.* it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

Once a recombinant cell that expresses the MRB gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). Alternatively, native MRB proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of MRB or other genes associated with the RB pathway. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

#### **Genetically modified animals**

Animal models that have been genetically modified to alter MRB expression may be used in *in vivo* assays to test for activity of a candidate RB modulating agent, or to further assess the role of MRB in a RB pathway process such as apoptosis or cell proliferation. Preferably, the altered MRB expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal MRB expression. The genetically modified animal may additionally have altered RB expression (*e.g.* RB knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice), cows, horses, goats, sheep, pigs, dogs and cats. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, *i.e.* mosaic animals (see, for example, techniques described by Jakobovits, 1994, *Curr. Biol.* 4:761-763.) or stably integrated into its germ line DNA (*i.e.*, in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster *et al.*, *Proc. Nat. Acad. Sci. USA* 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Pat. No. 4,873,191 by Wagner *et al.*, and Hogan, B., *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sandford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, *Science* (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic

insects see Berghammer A.J. *et al.*, A Universal Marker for Transgenic Insects (1999) Nature 402:370-371; for transgenic Zebrafish see Lin S., Transgenic Zebrafish, Methods Mol Biol. (2000);136:375-3830; for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, Experientia (1991) 47:897-905; for transgenic rats see Hammer *et al.*, Cell (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) Nature 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous MRB gene that results in a decrease of MRB function, preferably such that MRB expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse MRB gene is used to construct a homologous recombination vector suitable for altering an endogenous MRB gene in the mouse genome. Detailed methodologies for homologous recombination in mice are available (see Capecchi, Science (1989) 244:1288-1292; Joyner *et al.*, Nature (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, Science (1989) 244:1281-1288; Simms *et al.*, Bio/Technology (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene



Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*, PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) Science 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the transgene, and for sequential deletion of vector sequences in the same cell (Sun X *et al* (2000) Nat Genet 25:83-6).





In addition to the above-described genetically modified animals having altered MRB function, animal models having defective RB function (and otherwise normal MRB function), can be used in the methods of the present invention. For example, a mouse with defective RB function can be used to assess, *in vivo*, the activity of a candidate p53 modulating agent identified in one of the *in vitro* assays described below. Transgenic mice with defective RB function have been described in literature (Robanus-Maandag E et al. (1998) Genes Dev 12:1599-609; Windle, J. J. et al (1990) Nature 343: 665-669).

Preferably, the candidate RB modulating agent when administered to a model system with cells defective in RB function, produces a detectable phenotypic change in the model system indicating that the RB function is restored, i.e., the cells exhibit normal cell cycle progression.

### **Modulating Agents**

The invention provides methods to identify agents that interact with and/or modulate the function of MRB and/or the RB pathway. Modulating agents identified by the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the RB pathway, as well as in further analysis of the MRB protein and its contribution to the RB pathway. Accordingly, the invention also provides methods for modulating the RB pathway comprising the step of specifically modulating MRB activity by administering a MRB-interacting or -modulating agent.

As used herein, an "MRB-modulating agent" is any agent that modulated MRB function, for example, an agent that interacts with MRB to inhibit or enhance MRB activity or otherwise affect normal MRB function. MRB function can be affected at any level, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a preferred embodiment, the MRB - modulating agent specifically modulates the function of the MRB. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the MRB polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the MRB. These phrases also encompasses modulating agents that alter the interaction of the MRB with a binding partner, substrate, or cofactor

(e.g. by binding to a binding partner of an MRB, or to a protein/binding partner complex, and altering MRB function). In a further preferred embodiment, the MRB- modulating agent is a modulator of the RB pathway (e.g. it restores and/or upregulates RB function) and thus is also a RB-modulating agent.

Preferred MRB-modulating agents include small molecule compounds; MRB-interacting proteins, including antibodies and other biotherapeutics; and nucleic acid modulators such as antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19<sup>th</sup> edition.

#### **Small molecule modulators**

Small molecules are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more preferably less than 1,000, and most preferably less than 500. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the MRB protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for MRB-modulating activity. Methods for generating and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating

pathologies associated with the RB pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

### Protein Modulators

Specific MRB-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to the RB pathway and related disorders, as well as in validation assays for other MRB-modulating agents. In a preferred embodiment, MRB-interacting proteins affect normal MRB function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, MRB-interacting proteins are useful in detecting and providing information about the function of MRB proteins, as is relevant to RB related disorders, such as cancer (e.g., for diagnostic means).

An MRB-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with an MRB, such as a member of the MRB pathway that modulates MRB expression, localization, and/or activity. MRB-modulators include dominant negative forms of MRB-interacting proteins and of MRB proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous MRB-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3<sup>rd</sup>, Trends Genet (2000) 16:5-8).

An MRB-interacting protein may be an exogenous protein, such as an MRB-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) Antibodies, A

Laboratory Manual, Cold Spring Harbor Laboratory; Harlow and Lane (1999) Using antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). MRB antibodies are further discussed below.

In preferred embodiments, an MRB-interacting protein specifically binds an MRB protein. In alternative preferred embodiments, an MRB-modulating agent binds an MRB substrate, binding partner, or cofactor.

### ***Antibodies***

In another embodiment, the protein modulator is an MRB specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify MRB modulators. The antibodies can also be used in dissecting the portions of the MRB pathway responsible for various cellular responses and in the general processing and maturation of the MRB.

Antibodies that specifically bind MRB polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of MRB polypeptide, and more preferably, to human MRB. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Epitopes of MRB which are particularly antigenic can be selected, for example, by routine screening of MRB polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Natl. Acad. Sci. U.S.A. 78:3824-28; Hopp and Wood, (1983) Mol. Immunol. 20:483-89; Sutcliffe et al., (1983) Science 219:660-66) to the amino acid sequence of an MRB. Monoclonal antibodies with affinities of  $10^8 \text{ M}^{-1}$  preferably  $10^9 \text{ M}^{-1}$  to  $10^{10} \text{ M}^{-1}$ , or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of MRB or substantially purified fragments thereof. If MRB fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of an MRB protein. In a particular embodiment, MRB-

specific antigens and/or immunogens are coupled to carrier proteins that stimulate the immune response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

The presence of MRB-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding MRB polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

Chimeric antibodies specific to MRB polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat. Nos. 5,530,101, 5,585,089, 5,693,762, and 6,180,370).

MRB-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No.

4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989) 4:131-134). A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg -to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical

stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml to about 10 mg/ml.

Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

### **Nucleic Acid Modulators**

Other preferred MRB-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit MRB activity. Preferred nucleic acid modulators interfere with the function of the MRB nucleic acid such as DNA replication, transcription, translocation of the MRB RNA to the site of protein translation, translation of protein from the MRB RNA, splicing of the MRB RNA to yield one or more mRNA species, or catalytic activity which may be engaged in or facilitated by the MRB RNA.

In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to an MRB mRNA to bind to and prevent translation, preferably by binding to the 5' untranslated region. MRB-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

In another embodiment, the antisense oligomer is a phosphorothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits, each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphoramidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense

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Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. :7:187-95; US Pat. No. 5,235,033; and US Pat No. 5,378,841).

Alternative preferred MRB nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498).

Nucleic acid modulators are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Nucleic acid modulators are also used, for example, to distinguish between functions of various members of a biological pathway. For example, antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, an MRB-specific nucleic acid modulator is used in an assay to further elucidate the role of the MRB in the RB pathway, and/or its relationship to other members of the pathway. In another aspect of the invention, an MRB-specific antisense oligomer is used as a therapeutic agent for treatment of RB-related disease states.



### Assay Systems

The invention provides assay systems and screening methods for identifying specific modulators of MRB activity. As used herein, an "assay system" encompasses all the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the MRB nucleic acid or protein. In general, secondary assays further assess the activity of a MRB modulating agent identified by a primary assay and may confirm that the modulating agent affects MRB in a manner relevant to the RB pathway. In some cases, MRB modulators will be directly tested in a secondary assay.

In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising an MRB polypeptide or nucleic acid with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. binding activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates MRB activity, and hence the RB pathway. The MRB polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

### **Primary Assays**

The type of modulator tested generally determines the type of primary assay.

#### ***Primary assays for small molecule modulators***

For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially

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purified protein (either endogenous or recombinantly produced), partially purified or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding), transcriptional activity (*e.g.*, using a reporter gene), enzymatic activity (*e.g.*, via a property of the substrate), activity of second messengers, immunogenicity and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular molecular event detected.

Cell-based screening assays usually require systems for recombinant expression of MRB and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when MRB-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the MRB protein may be assayed by various known methods such as substrate processing (*e.g.* ability of the candidate MRB-specific binding agents to function as negative effectors in MRB-expressing cells), binding equilibrium constants (usually at least about  $10^7 \text{ M}^{-1}$ , preferably at least about  $10^8 \text{ M}^{-1}$ , more preferably at least about  $10^9 \text{ M}^{-1}$ ), and immunogenicity (*e.g.* ability to elicit MRB specific antibody in a heterologous host such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of a MRB polypeptide, a fusion protein thereof, or to cells or membranes bearing the polypeptide or fusion protein. The MRB polypeptide can be full length or a fragment thereof that retains functional MRB activity. The MRB polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The MRB polypeptide is preferably human MRB, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay

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detects candidate agent-based modulation of MRB interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has MRB-specific binding activity, and can be used to assess normal MRB gene function.

Suitable assay formats that may be adapted to screen for MRB modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, *Curr Opin Chem Biol* (1998) 2:597-603; Sundberg SA, *Curr Opin Biotechnol* 2000, 11:47-53). In one preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, *Nat Struct Biol* (2000) 7:730-4; Fernandes PB, *supra*; Hertzberg RP and Pope AJ, *Curr Opin Chem Biol* (2000) 4:445-451).

A variety of suitable assay systems may be used to identify candidate MRB and RB pathway modulators (*e.g.* U.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); U.S. Pat. No. 6,114,132 (phosphatase and protease assays), among others). Specific preferred assays are described in more detail below.

Preferred MRBs are ATPases and RNA binding proteins.

RNA folds into a myriad of tertiary structures that are responsible for its diverse functions in cells. In most instances, RNA is associated with RNA-binding proteins (RBPs) that protect, stabilize, package or transport RNA, mediate RNA interactions with other biomolecules or act catalytically on RNA. The structural information obtained for RNA alone and RNA-protein complexes has elucidated a variety of RNA tertiary structures and diverse modes for RNA-protein interaction. The specific interaction of proteins with highly structured RNAs makes it possible to target unique RNA motifs with small molecules, thus making RNA an interesting target for therapeutic intervention. Assays for RNA binding or processing may be based on homogeneous scintillation proximity (Liu J, *et al.*, *Anal Biochem* 2001 289:239-245), chemiluminescence (Mazumder A, *Nucleic Acids Res* 1998 26:1996-2000), gel shift (Stull RA, *et al.*, *Antisense Nucleic Acid Drug Dev* 1996 6:221-228; U.S. Pat. No: 6004749).

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Assays for ATPase activity are known in the art, and are described, for example, in Blackburn et al (Blackburn CL, et al., (1999) J Org Chem 64:5565-5570). The ATPase assay is performed using the EnzCheck ATPase kit (Molecular Probes). The assays are performed using an Ultraspec spectrophotometer (Pharmacia), and the progress of the reaction are monitored by absorbance increase at 360 nm. Microtubules (1.7 mM final), kinesin (0.11 mM final), inhibitor (or DMSO blank at 5% final), and the EnzCheck components (purine nucleotide phosphorylase and MESG substrate) are premixed in the cuvette in a reaction buffer (40 mM PIPES pH 6.8, 5 mM paclitaxel, 1 mM EGTA, 5 mM MgCl<sub>2</sub>). The reaction is initiated by addition of MgATP (1 mM final).

**Apoptosis assays.** Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., et al., 1998, Blood 15:4730-41). An apoptosis assay system may comprise a cell that expresses an MRB, and that optionally has defective RB function (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate RB modulating agents. In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether MRB function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express MRB relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the MRB plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

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**Cell proliferation and cell cycle assays.** Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, *Int. J. Cancer* 38, 369; Campana *et al.*, 1988, *J. Immunol. Meth.* 107, 79), or by other means.

Cell Proliferation may also be examined using [<sup>3</sup>H]-thymidine incorporation (Chen, J., 1996, *Oncogene* 13:1395-403; Jeoung, J., 1995, *J. Biol. Chem.* 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [<sup>3</sup>H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter).

Cell proliferation may also be assayed by colony formation in soft agar (Sambrook *et al.*, *Molecular Cloning*, Cold Spring Harbor (1989)). For example, cells transformed with MRB are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray JW *et al.* (1986) *Int J Radiat Biol Relat Stud Phys Chem Med* 49:237-55). Cells transfected with an MRB may be stained with propidium iodide and evaluated in a flow cytometer (available from Becton Dickinson).

Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses an MRB, and that optionally has defective RB function (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no test agent is added, identify candidate RB modulating agents. In some embodiments of the invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using another assay system such as a cell-free assay system. A cell proliferation assay may also be used to test whether MRB function plays a direct role in cell proliferation or cell cycle. For example, a cell proliferation or cell cycle assay may be performed on cells that over-

or under-express MRB relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the MRB plays a direct role in cell proliferation or cell cycle.

**Angiogenesis.** Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses an MRB, and that optionally has defective RB function (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate RB modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether MRB function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express MRB relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the MRB plays a direct role in angiogenesis.

**Hypoxic induction.** The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glycolytic enzymes and VEGF. Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with MRB in hypoxic conditions (such as with 0.1% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic

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conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses an MRB, and that optionally has a mutated RB (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate RB modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether MRB function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express MRB relative to wild type cells. Differences in hypoxic response compared to wild type cells suggests that the MRB plays a direct role in hypoxic induction.

**Cell adhesion.** Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents. Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2× final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF, and allowed to adhere to the monolayers in the presence of candidate agents.

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Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., *Bioconjug Chem.* 2001 May-Jun;12(3):346-53).

**Cell Migration.** An invasion/migration assay (also called a migration assay) tests the ability of cells to overcome a physical barrier and to migrate towards pro-angiogenic signals. Migration assays are known in the art (e.g., Paik JH et al., 2001, *J Biol Chem* 276:11830-11837). In a typical experimental set-up, cultured endothelial cells are seeded onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The matrix generally simulates the environment of the extracellular matrix, as described above. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic stimuli. Migration is generally assayed after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Migration is assessed as the number of cells that crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific, South San Francisco, CA), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and migration is detected using fluorescent readings, for instance using the Falcon HTS FluoroBlok (Becton Dickinson). While some migration is observed in the absence of stimulus, migration is greatly increased in response to pro-angiogenic factors. As described above, a preferred assay system for migration/invasion assays comprises testing an MRB's response to a variety of pro-angiogenic factors, including tumor angiogenic and inflammatory angiogenic agents, and culturing the cells in serum free medium.



### ***Primary assays for antibody modulators***

For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the MRB protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for detecting MRB-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

### ***Primary assays for nucleic acid modulators***

For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance MRB gene expression, preferably mRNA expression. In general, expression analysis comprises comparing MRB expression in like populations of cells (*e.g.*, two pools of cells that endogenously or recombinantly express MRB) in the presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that MRB mRNA expression is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with specific antibodies or antisera directed against either the MRB protein or specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

### **Secondary Assays**

Secondary assays may be used to further assess the activity of MRB-modulating agent identified by any of the above methods to confirm that the modulating agent affects MRB in a manner relevant to the RB pathway. As used herein, MRB-modulating agents

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encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with MRB.

Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express MRB) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate MRB-modulating agent results in changes in the RB pathway in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the RB or interacting pathways.

#### *Cell-based assays*

Cell based assays may use a variety of mammalian cell lines known to have defective RB function (*e.g.* SAOS-2 osteoblasts, BT549 breast cancer cells, and C33A cervical cancer cells, among others, available from American Type Culture Collection (ATCC), Manassas, VA). Cell based assays may detect endogenous RB pathway activity or may rely on recombinant expression of RB pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

#### *Animal Assays*

A variety of non-human animal models of normal or defective RB pathway may be used to test candidate MRB modulators. Models for defective RB pathway typically use genetically modified animals that have been engineered to mis-express (*e.g.*, over-express or lack expression in) genes involved in the RB pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

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In a preferred embodiment, RB pathway activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal RB are used to test the candidate modulator's affect on MRB in Matrigel® assays. Matrigel® is an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4° C, but rapidly forms a solid gel at 37° C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells which over-express the MRB. The mixture is then injected subcutaneously(SC) into female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral (PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit). Hemoglobin content of the gel is found to correlate the degree of neovascularization in the gel.

In another preferred embodiment, the effect of the candidate modulator on MRB is assessed via tumorigenicity assays. In one example, xenograft human tumors are implanted SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the MRB endogenously are injected in the flank,  $1 \times 10^5$  to  $1 \times 10^7$  cells per mouse in a volume of 100  $\mu$ L using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100 mg. Candidate modulator is delivered IV, SC, IP, or PO by bolus administration. Depending upon the pharmacokinetics of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors maybe utilized for biomarker identification or further analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

**Diagnostic and therapeutic uses**

Specific MRB-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the RB pathway, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating the RB pathway in a cell, preferably a cell pre-determined to have defective or impaired RB function (e.g. due to overexpression, underexpression, or misexpression of RB, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates MRB activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the RB function is restored. The phrase "function is restored", and equivalents, as used herein, means that the desired phenotype is achieved, or is brought closer to normal compared to untreated cells. For example, with restored RB function, cell proliferation and/or progression through cell cycle may normalize, or be brought closer to normal relative to untreated cells. The invention also provides methods for treating disorders or disease associated with impaired RB function by administering a therapeutically effective amount of an MRB -modulating agent that modulates the RB pathway. The invention further provides methods for modulating MRB function in a cell, preferably a cell pre-determined to have defective or impaired MRB function, by administering an MRB -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired MRB function by administering a therapeutically effective amount of an MRB -modulating agent.

The discovery that MRB is implicated in RB pathway provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in the RB pathway and for the identification of subjects having a predisposition to such diseases and disorders.

Various expression analysis methods can be used to diagnose whether MRB expression occurs in a particular sample, including Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR, and microarray analysis. (e.g., Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-

47). Tissues having a disease or disorder implicating defective RB signaling that express an MRB, are identified as amenable to treatment with an MRB modulating agent. In a preferred application, the RB defective tissue overexpresses an MRB relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial MRB cDNA sequences as probes, can determine whether particular tumors express or overexpress MRB. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of MRB expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

Various other diagnostic methods may be performed, for example, utilizing reagents such as the MRB oligonucleotides, and antibodies directed against an MRB, as described above for: (1) the detection of the presence of MRB gene mutations, or the detection of either over- or under-expression of MRB mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of MRB gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by MRB.

Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease or disorder in a patient that is associated with alterations in MRB expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for MRB expression; c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of the disease or disorder. Preferably, the disease is cancer. The probe may be either DNA or protein, including an antibody.

## EXAMPLES

The following experimental section and examples are offered by way of illustration and not by way of limitation.

### I. Drosophila cell RB screen

RNA interference (RNAi) was used to create Rbf-deficient cultured Drosophila cells (Schneider S2 cells (Schneider, I. (1972) J. Embryol. Exp. Morph. 27, 363), adapted to

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serum-free media, from Invitrogen Corp., Carlsbad, CA). Cells were treated for 3 days with Rbf double stranded RNA (dsRNA) or a control dsRNA representing sequences from an EGFP luciferase cDNA. Following pretreatment with Rbf or control dsRNA, cells were plated in 96-well format and dsRNA representing approximately 6000 different *Drosophila* genes were added to individual wells. A cell proliferation assay (ProCheck™ assay – Serological Corporation, Norcross, GA) was used to quantify cell viability after a 96-hour incubation. For each of the greater than 6000 dsRNA sequences tested in this manner, cell viability data was obtained on Rbf-deficient cells (Rbf dsRNA-treated) and control cells (EGFP luciferase dsRNA-treated). Comparison of this data for each dsRNA identified dsRNA sequences that preferentially reduced the viability of Rbf-deficient cells.

## II. Analysis of Table 1

BLAST analysis (Altschul et al., *supra*) was employed to identify Targets from *Drosophila* modifiers. The columns “MRB symbol”, and “MRB name aliases ” provide a symbol and the known name abbreviations for the Targets, where available, from Genbank. “MRB RefSeq\_NA or GI\_NA”, “MRB GI\_AA”, “MRB NAME”, and “MRB Description” provide the reference DNA sequences for the MRBs as available from National Center for Biology Information (NCBI), MRB protein Genbank identifier number (GI#), MRB name, and MRB description, all available from Genbank, respectively. The length of each amino acid is in the “MRB Protein Length” column.

Protein sequences of *Drosophila* modifiers of RB from screen (Example I), are represented in the “Modifier GI\_AA” column by GI#.

## III. High-Throughput In Vitro Fluorescence Polarization Assay

Fluorescently-labeled MRB peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of MRB activity.

#### IV. High-Throughput In Vitro Binding Assay.

<sup>33</sup>P-labeled MRB peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl<sub>2</sub>, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate RB modulating agents.

#### V. Immunoprecipitations and Immunoblotting

For coprecipitation of transfected proteins,  $3 \times 10^6$  appropriate recombinant cells containing the MRB proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at  $15,000 \times g$  for 15 min. The cell lysate is incubated with 25  $\mu$ l of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

#### VI. Expression analysis

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All cell lines used in the following experiments are NCI (National Cancer Institute) lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues are obtained from Impath, UC Davis, Clontech, Stratagene, and Ambion.

TaqMan analysis is used to assess expression levels of the disclosed genes in various samples.

RNA is extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/ $\mu$ l. Single stranded cDNA is then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of Applied Biosystems (Foster City, CA).

Primers for expression analysis using TaqMan assay (Applied Biosystems, Foster City, CA) are prepared according to the TaqMan protocols, and the following criteria: a) primer pairs are designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product.

Taqman reactions are carried out following manufacturer's protocols, in 25  $\mu$ l total volume for 96-well plates and 10  $\mu$ l total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis is prepared using a universal pool of human cDNA samples, which is a mixture of cDNAs from a wide variety of tissues so that the chance that a target will be present in appreciable amounts is good. The raw data are normalized using 18S rRNA (universally expressed in all tissues and cells).

For each expression analysis, tumor tissue samples are compared with matched normal tissues from the same patient. A gene is considered overexpressed in a tumor when the level of expression of the gene is 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue is not available, a universal pool of cDNA samples is used instead. In these cases, a gene is considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the average of all normal samples from the same tissue type is greater than 2 times the standard deviation of all normal samples (i.e.,  $\text{Tumor} - \text{average}(\text{all normal samples}) > 2 \times \text{STDEV}(\text{all normal samples})$ ).



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A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression analysis as described above, by antibody directed to the gene target, or by any other available detection method.

**WHAT IS CLAIMED IS:**

1. A method of identifying a candidate RB pathway modulating agent, said method comprising the steps of:
  - (a) providing an assay system comprising a purified MRB polypeptide or nucleic acid or a functionally active fragment or derivative thereof;
  - (b) contacting the assay system with a test agent under conditions whereby, but for the presence of the test agent, the system provides a reference activity; and
  - (c) detecting a test agent-biased activity of the assay system, wherein a difference between the test agent-biased activity and the reference activity identifies the test agent as a candidate RB pathway modulating agent.
2. The method of Claim 1 wherein the assay system comprises cultured cells that express the MRB polypeptide.
3. The method of Claim 2 wherein the cultured cells additionally have defective RB function.
4. The method of Claim 1 wherein the assay system includes a screening assay comprising a MRB polypeptide, and the candidate test agent is a small molecule modulator.
5. The method of Claim 4 wherein the assay is a binding assay.
6. The method of Claim 1 wherein the assay system is selected from the group consisting of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay system, and a hypoxic induction assay system.
7. The method of Claim 1 wherein the assay system includes a binding assay comprising a MRB polypeptide and the candidate test agent is an antibody.

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8. The method of Claim 1 wherein the assay system includes an expression assay comprising a MRB nucleic acid and the candidate test agent is a nucleic acid modulator.
9. The method of claim 8 wherein the nucleic acid modulator is an antisense oligomer.
10. The method of Claim 8 wherein the nucleic acid modulator is a PMO.
11. The method of Claim 1 additionally comprising:
  - (d) administering the candidate RB pathway modulating agent identified in (c) to a model system comprising cells defective in RB function and, detecting a phenotypic change in the model system that indicates that the RB function is restored.
12. The method of Claim 11 wherein the model system is a mouse model with defective RB function.
13. A method for modulating a RB pathway of a cell comprising contacting a cell defective in RB function with a candidate modulator that specifically binds to a MRB polypeptide, whereby RB function is restored.
14. The method of claim 13 wherein the candidate modulator is administered to a vertebrate animal predetermined to have a disease or disorder resulting from a defect in RB function.
15. The method of Claim 13 wherein the candidate modulator is selected from the group consisting of an antibody and a small molecule.
16. The method of Claim 1, comprising the additional steps of:
  - (d) providing a secondary assay system comprising cultured cells or a non-human animal expressing MRB ,

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(e) contacting the secondary assay system with the test agent of (b) or an agent derived therefrom under conditions whereby, but for the presence of the test agent or agent derived therefrom, the system provides a reference activity; and

(f) detecting an agent-biased activity of the second assay system, wherein a difference between the agent-biased activity and the reference activity of the second assay system confirms the test agent or agent derived therefrom as a candidate RB pathway modulating agent, and wherein the second assay detects an agent-biased change in the RB pathway.

17. The method of Claim 16 wherein the secondary assay system comprises cultured cells.

18. The method of Claim 16 wherein the secondary assay system comprises a non-human animal.

19. The method of Claim 18 wherein the non-human animal mis-expresses a RB pathway gene.

20. A method of modulating RB pathway in a mammalian cell comprising contacting the cell with an agent that specifically binds a MRB polypeptide or nucleic acid.

21. The method of Claim 20 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with the RB pathway.

22. The method of Claim 20 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.

23. A method for diagnosing a disease in a patient comprising:

- (a) obtaining a biological sample from the patient;
- (b) contacting the sample with a probe for MRB expression;
- (c) comparing results from step (b) with a control;
- (d) determining whether step (c) indicates a likelihood of disease.

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24. The method of claim 23 wherein said disease is cancer.

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**ABSTRACT OF THE DISCLOSURE**

Human MRB genes are identified as modulators of the RB pathway, and thus are therapeutic targets for disorders associated with defective RB function. Methods for identifying modulators of RB, comprising screening for agents that modulate the activity of MRB are provided.

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# Nucleic Acid and Polypeptide sequences

>gi|21361143|ref|NM\_002804.2| Homo sapiens proteasome (prosome, macropain) 26S subunit, ATPase, 3 (PSMC3), mRNA  
GGCAGGAGGATTCGCTGCTCCGACGACGGCCGGAGCTGGTCCGGTCAAGAGTCGGGATTGTGGGGAGA  
GGTTTTCCTACTGGTCAAGAGAAGGCTTTAAGAAAGACGGTATTAATCTCCCGTTGCGGCTCCCGCCTGGT  
CCCATCTTCTGCCCGCTCCTCCAGGAAATGAATCTGCTGCCGAATATTGAGAGTCCAGTGACTCGGCAGG  
AGAAGATGGCGACCGTGTGGGATGAGGCCGAGCAAGATGGAATTGGGGAGGAGGTGCTCAAGATGTCCAC  
GGAGGAGATCATCCAGCGCACACGGCTGCTGGACAGTGAGATCAAGATCATGAAGAGTGAAGTGTGAGA  
GTCACCCATGAGCTCCAAGCCATGAAGGACAAGATAAAAAGAGAACAGTGAGAAAATCAAAGTGAACAAGA  
CCCTGCCGTACCTTGTCTCCAACGTCATCGAGCTCCTGGATGTTGATCCTAATGACCAAGAGGAGGATGG  
TGGCAATATTGACCTGGACTCCCAGAGGAAGGGCAAGTGTGCTGTGATCAAAACCTCTACACGACAGACG  
TACTTCTTCTGTGATTGGGTTGGTGGATGCTGAAAAGCTAAAGCCAGGAGACCTGGTGGGTGTGAACA  
AAGACTCCTATCTGATCCTGGAGACGCTGCCCACAGAGTATGACTCGCGGTGAAGGCCATGGAGGTAGA  
CGAGAGGCCACCGAGCAATACAGTGACATTGGGGGTTTGGACAAGCAGATCCAGGAGCTGGTGGAGGCC  
ATTGTCTTGCCAATGAACCACAAGGAGAAGTTTGAAGACTTGGGGATCCAACCTCCAAAAGGGGTGCTGA  
TGTATGGGCCCCCAGGGACGGGGAAGACCCCTCCTGGCCCGGGCCTGTGCCGACAGACTAAGGCCACCTT  
CCTAAAGCTGGCTGGCCCCAGCTGGTGCAGATGTTTATTGGAGATGGTGGCAAGCTAGTCCGGGATGCC  
TTTGCCCTGGCCCAAGGAGAAAGCGCCCTCTATCATCTTCATTGATGAGTTGGATGCCATCGGCACCAAGC  
GCTTTGACAGTGAGAAGGCTGGGGACCGGGAGGTGCAGAGGACAATGCTGGAGCTTCTGAACCAGCTGGA  
TGGCTTCCAGCCCAACACCCAAGTTAAGGTAATTGCAGCCACAAACAGGGTGGACATCCTGGACCCCGCC  
CTCCTCCGCTCGGGCCCGCCTTGACCGCAAGATAGAGTTCCCGATGCCCAATGAGGAGGCCCGGGCAGAA  
TCATGCAGATCCACTCCCGAAAGATGAATGTGAGTGTGCTGTGGAGGCGGGCATGATCGCACTGCGCAGGGGT  
AGATGACTTCAATGGGGCCAGTGCAAGGCTGTGTTGTTGGAGGCGGGCATGATCGCACTGCGCAGGGGT  
GCCACGGAGCTCACCCACGAGGACTACATGGAAGGCATCCTGGAGGTGCAGGCCAAGAAGAAAGCCAACC  
TACAATACTACGCCTAGGGCACACAGGCCAGCCCCAGTCTCACGGCTGAAGTGCGCAATAAAAGATGGTT  
TAGGGTCCCTGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

>gi|20533869|ref|XM\_116191.1| Homo sapiens similar to ribosomal protein S6 (LOC205865), mRNA  
ATGAAGCTGAACAGCTCCTTCTTAGCCATTGGCTGCCAGAACTCATTAAAGTGAAGATGAACGCAAAC  
TTCGTACTTTTATGAGAAGCATATGGCCACAGAAATGTCTGCTTATGCTCTGGGTGAAGAATGGAAGGG  
TTATGTGTTCGAATCAGTGGTGGGAACAACAAGGTTTCTTGTGTTGGTGGGAACAACAAGCAG  
GAAGATGATGTCTGCCAGTATGTTGTAAGAAAGCCCTTAAACAAGAAGGTAAGAAACCTAGGACCAAG  
CACCAAGATTACAGCATCTTGTACTCCACGTGTCTGCAGCACAAACAGCAGCCTATTGCTCTGAAGAA  
GCAGTGTACTAAGAAAAACAGGGAAGAGGCTGCAGAATATGCTAAACTTTCGGCCAAGAGAATGAAGGAG  
GCTAAAGAGAAGCGCAAGGAACAAGTTGTGAAGAGACGCAGACTTTCCTCTCTGCGAGCTTCTGCTTCTA  
AGTCTGAATCTAGTCAGGAAAAAGATTTTTGTAGTAACAAATAA

>gi|17448284|ref|XM\_070395.1| Homo sapiens similar to Rim2 protein (LOC137397), mRNA  
ATGAAGGAATTCATTCTTTTAAATAGCATAAACTGCATCAGCAGTTTGAAATGTATAAAGAGCAGGTAA  
AGAAGATGGGAGAAGAATCACAGCAACAGCAAGAACAGAAGGGTGATGCGCCAACCTGTGGTATCTGCCA  
CAAAACAAAGTTTGTGATGGATGTGGCCATAACTGTTCATATTGCCAAACAAAGTTCTGTGCTCGTTGT  
GGAGGTGAGTGTCTTACGCTCAAACAAGGTTATGTGGGTATGTAATTTGTGCCGAAAACAACAAGAAA  
TCCTCACTAAATCAGGAGCATGGTTTTTATAATAGTGGATCTAATACACCACAGCAACCTGATCAAAAGGT  
TCTTCGAGGGCTAAGAAATGAGGAGGCACCTCAGGAGAAGAAACCAAACTACATGAGCAGACCCAGTTC  
CAAGGACCCTCAGCCACTGGCTGCCAGAACTCATTGAAGTGGATGATAAACGCAAACTCTGCATTTTTT  
ATGAGAAGCATATGGCCACAGAAGTTGCTGCTGATGCTCTGGTTGAAGAATGGAAGGGTTATGTGGTCTT  
AATCAGTGGTGGGAATGACAAACAATGGTTCCCCATGAAGAGGGGTGCTTTGATTTCATGGCCGTGTCCGC  
CTGCTAATGAGAGATAAAAAATATCCAGGATTGACTGATACGATGCCTCATCACCTGGGGCCCCAAAA  
GAGCTAGCAGATCCACAACTTTTCAATCTCTCTAAAGAAGATGATGTCCACCAGTATGTTGTAAGAAA  
GCCTTTAAACAAGAAGGTAAGAAACCTAGGACCAAGCACCCAAATTCAGCGTCTTATTACTCCATGT  
GTCCTGCAGCACAAATGGTGCCATATTGCTCTGGAGAAGCAGCATACTAAGAAAAATTAG

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>gi|17458724|ref|XM\_061988.1| Homo sapiens similar to ribosomal protein S6 (LOC120318), mRNA  
 ATGAAGCTGAACATCTTCCTGGCCACTGCCTGCCAGAACTCATTGAAGTGGACAATGAACACAAACTTC  
 ATACTTTTATGAGAAGTGTATGGCCACAGAAGTTGCTGCTGATGCTCTGGGTAAAGAATGGAAGGGTTA  
 TGTGGGTGCTCTTGACCCATGGCCATGTCCACCTGCTGCTGAATAAAAGGCATGCCTGTTACAGACCAAGA  
 AGAACTGGAAAAAGAAAGCAAAAATCTGCACGTGGTTGCATTGTGGATGCCAATCTGAGCATTCTCAACT  
 TGGTTATTGTAAAAAAGGAGAGAAGGATATTCTGGACTGACTGATACTATGGTGCCTTGTTCATCTCGG  
 GCCAAAAGAGCTAGCAGAATCTGCAAACCTTTTCAGTCTCTCTGAAGATGATGATGTCCACCAGTGTGTT  
 GTAAGAAAGGCTTTAAACAAAGAAGGTAAGAAACCTAGGACCAAAGCACCACGACTAAGTATCTTGTTA  
 CTCACATGTTCTGCAGCACGAATGGTGGCATATTGCTCTGAAGAAACAGAGAATTAAGGAAAATAAGGA  
 AGTAGCTGCAGAAATATGCTAAACTTTTGGCCAAGAGAATGAAGGAGGCTAAAGAGAAGCATCAAGAACAG  
 ATTGCCAAGAGACGCAGACTGTCTCTCTTTGTTTGGGTGAAGAATAATCTTTGTTTGAACAGAGGC  
 ATTTGATAGCTACTGGAGCTAATTTCTGTATCACTGTGTGGACATAA

>gi|18579096|ref|XM\_090253.1| Homo sapiens similar to ribosomal protein S6 (LOC160369), mRNA  
 ATGAAGCTGAACATCTCCCTCCCGGTCACTGGCTGCCAGAACTCATTGAAGTGGATGATGAATGCAAAAC  
 TTCGTACTTTTTATGAGAAGCTTATGGCCACAGAAGTTGCTGCTGACACTCTGGGTGAAGAATGGAAGG  
 TTATGTGGTCCGAATCAGTGGTGGGAACAACAACAAGGTTTCCCATGAAGCAGGGTGTCTTGACCCAT  
 GGCCGTGTCCACCTGCTACTGAGTAAGGGGCATTCCTGTTACAGACCAAGGAGAACTGGAGAAAGAAAGA  
 GAAAATCAGTTTCGTGGTTGCATAGTGGATGCCAATCTGAGCATTCTCAACTTGATTTATGTTAAAAAAA  
 AAAAAAAGTAAAGAAGGATATTCTGGACTGACTGATACATACTATGGTGCCTTGTGCGCTGGGGCCCAAAAA  
 CCTAGCAGAATCTGCAAACCTTTCCAATCTCTCTGAAGAAGATGATGTCCGCCAGTATGTTGTAAGAAAGC  
 CCTCAAACAAAGGAGGTAAGAAACCTAGGACCAAAGCACCAGATTTCAGCATCTTGTACTCCACATTT  
 CCTGCAGCACAAAGGGCAGCATATTGCTCTGAAGAAGCCGTGTACTAAGAAAAATAAGGAAGAGGCTGCA  
 GAATATGCTAAACTTTTAGGCAAGGAATGAAGGAGGCTAAAGAGAAGCGCCAGGAACAAATTGCCGAAGA  
 GACACAGACTTTCTCTCTGTGA

>gi|17158043|ref|NM\_001010.2| Homo sapiens ribosomal protein S6 (RPS6), mRNA  
 CCTCTTTTCCGTGGCGCCTCGGAGGCGTTTCAGCTGCTTCAAGATGAAGCTGAACATCTCCTTCCAGCCA  
 CTGGCTGCCAGAACTCATTGAAGTGGACGATGAACGCAAACTTCGTACTTTCTATGAGAAGCGTATGGC  
 CACAGAAGTTGCTGCTGACGCTCTGGGTGAAGAATGGAAGGGTTATGTGGTCCGAATCAGTGGTGGGAAC  
 GACAAACAAGGTTTCCCATGAAGCAGGGTGTCTTGACCCATGGCCGTGTCCGCTGCTACTGAGTAAGG  
 GGCAATCTCTGTTACAGACCAAGGAGAACTGGAGAAAGAAAGAGAAAATCAGTTTCGTGGTTGCATTGTGGA  
 TGCAAATCTGAGCGTTCTCAACTTGGTTATTTGTAAGAAAGGAGAGAAGGATATTCTGGACTGACTGAT  
 ACTACAGTGCCTCGCCGCTTGGGCCCCAAAAGAGCTAGCAGAATCCGCAAACTTTTCAATCTCTTAAG  
 AAGATGATGTCCGCCAGTATGTTGTAAGAAAGCCCTTAAATAAAGAAGGTAAGAAACCTAGGACCAAAGC  
 ACCCAAGATTTCAGCGTCTTGTACTCCACGTGTCTGCAGCACAAACGGCGGCGTATTGCTCTGAAGAAG  
 CAGCGTACCAAGAAAAATAAGAAAGAGGCTGCAGAATATGCTAAACTTTTGGCCAAGAGAATGAAGGAGG  
 CTAAGGAGAAGCGCCAGGAACAAATTGCCGAAGAGACGCAGACTTTCCTCTCTGCGAGCTTCTACTTCTAA  
 GTCTGAATCCAGTCAGAAATAAGATTTTTTGAGTAACAAATAAATAAGATCAGACTCTG

>gi|17474555|ref|XM\_062542.1| Homo sapiens similar to 40S ribosomal protein S6 (Phosphoprotein NP33) (LOC121253), mRNA  
 ATGAAGCTGAACATCTCCTTCCCCGCCACCGGCTGTCAGAAGCTCATCGAGGTGGATGACGAGCGCAAGC  
 TCCGCACCTTCTATGAGAAGCGCATGGCCACGGAAGTAGCCGCTGATGCTCTTGGTGAAGAGTGAAGGG  
 TTATGTGGTCCGGATCAGCGGTGGGAATGACAAGCAAGGGTTTCCCATGAAGCAAGGTGTTCTGACCCAT  
 GGCAGAGTGCCTGCTGTTGAGTAAGGGGCTTCTCTCAGTGTCTCAACTTGGTCATTGTTAAAGAAAGG  
 AGAGAAGGATATTCTGGACTGACAGACACTACTGTGCTCGTGGTGGGACCTAAAGGGGCTAGTAGA  
 ATCCGCAAGCTTTTAAATCTCTCAAAGAAGATGATGTCCGCCAGTATGTTGTGAGGAAGCCCTTAAACA  
 AAGAAGGTAAGAAGCCCAGGACCAAAGCACCAGATTTCAGCGACTTGTACTCTCGTGTCTGCAACA  
 CAAACGCCGACGTATTGCTCTGAAGAAGCAACGCCTAAGAAGAACAAGGAGGAGGCTGCAGAATACGCT  
 AAACCTTTTGGCCAAGAGAATGAAGGAAGCCAAAGAAAAGCGCCAGGAACAGATTGCCAAGAGACGTAGGC



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TGTCCTCACTGAGAGCTTCTACTTCTAAGTCTGAGTCCAGTCAAAAATGA

>gi|20552075|ref|XM\_115132.1| Homo sapiens similar to 40S ribosomal protein S6 (Phosphoprotein NP33) (LOC196391), mRNA  
 ATGAAGCTGAACATCTTCCCAGCCGCTGGCTGCCAGAAGCTCATTGCAGTGGACGATGAACACAAACTTC  
 GTAGTTTGTATGAGATGCCTGTGGCCACAGAAGTTGCTGTCTGATGCTCTCGTGAAGCATGGAAGGGTTCT  
 GTGGTCTGGATCAGTGGTGGGAACGACAAACAAGCCTTCCCATGAAGCAGGAAGAGCTAGCAGAATCCGT  
 AGACTTTTCAATGTCTCTAAAGAAGACGACGTCTTCTTTAGACGAAGAAGAAGACGTCTAAATGTTGAA  
 TGTGAAATGTTTCAATGTTAA

>gi|21361144|ref|NP\_002795.2| proteasome (prosome, macropain) 26S subunit, ATPase, 3 [Homo sapiens]  
 MNLLPNIESPVTRQEKMATVWDEAEQDGIGEEVLKMSTEEIIQTRRLDSEIKIMKSEVLRVTHELQAMK  
 DKIKENSEKIKVNKTLPYLVSNVIELLDVDPNDQEEDGANIDLDSQRKKGCAVIKTSTRQTYFLPVIGLV  
 DAEKLPKPGDLVGVNKSYSILETLPTFYDSRVKAMEVDERPTEQYSDIGGLDKQIQELVEAIVLPMNHKE  
 KFENLGIQPPKGVLMYGPPTGKTLARACAAQTATFLKLAGPQLVQMFIGDGAQLVRDAFALAKEKAP  
 SIIIFIDELDAIGTKRFDSEKAGDREVQRTMLELLNQLDGFPNTQVKVIAATNRVDILDPALLRSGRLDR  
 KIEFPMPNNEEARARIMQIHSRKMNVSPDVNYEELARCTDDFNGAQCKAVCVEAGMIALRRGATELTHEDY  
 MEGILEVQAKKANLQYYA

>gi|20533870|ref|XP\_116191.1| similar to ribosomal protein S6 [Homo sapiens]  
 MKLNSSFLAIGCQKLIKVEDERKLRTFYEKHMATEIAAYALGEEWKGYVVRISGGNNKQGFLFVGGNNKQ  
 EDDVCQYVVRKPLNKEGKKPRTKAPKIQHLVTPRVLQHKQPIALKKQCTKKNREEAAEYAKLSAKRMKE  
 AKEKRKEQVVKRRRLSSLRASAKSESSQEKDFLSNK

>gi|17448285|ref|XP\_070395.1| similar to Rim2 protein [Homo sapiens]  
 MKEFFIFNSIKLHQQFEMYKEQVKMGEESSQQQEQKGDAPTCGICHKTKFADGCGHNC SYCQTKFCARC  
 GGRVSLRSNKMVMWCNLCRKQOEILTKSGAWFYNSGNTPPQPDQKVLRLRNEEAPQEKKPKLHEQTQF  
 QGPSATGCQKLIIEVDKRLCTFYEKHMATEVAADALVEEWKGYVVLISGGNDKQWFPMKRGVLIHGRVR  
 LLMRDKNIPGLTDTTTPHHLGPKRASRIHKLFLNLSKEDDVHQYVVRKPLNKEGKKPRTKAPKIQRLITPC  
 VLQHKWCHIALEKQHTKKN

>gi|17458725|ref|XP\_061988.1| similar to ribosomal protein S6 [Homo sapiens]  
 MKLNI FLATACQKLIIEVDNEHKLHTFYEKCMATEVAADALGKEWKGYVGVLTGHVHLLLNKRHACYRPR  
 RTGKRKQKSARGCIVDANLSILNLVIVKKGEDIPLGLTDMVPCHLGPKRASRICKLFLSLSEDDDVHQCV  
 VRKALNKEGKKPRTKAPTKYLVTPLVQHEWVHIALKKQRIKENKEVAEYAKLLAKRMKEAKEKHQEQ  
 IAKRRRLSSLCLWVKNNLCLEQRHLIATGANFCITVWT

>gi|18579097|ref|XP\_090253.1| similar to ribosomal protein S6 [Homo sapiens]  
 MKLNI SFPTGCQKLIIEVDDECKLRTFYEKLMATEVAADTLGEEWKGYVVRISGGNNKQGFPMKQGVLT  
 GRVHLLLSKGHSCYRPRRTGERKRKSVRGCIVDANLSILNLIIVKKKKVKKDIPLGLTDMVPCRLGPKK  
 ASRICKLSNLSEDDVRQYVVRKPSNKGKKPRTKAPKIQHLVTPHFLQHKGQHIALKKPCTKKNKEEAA  
 EYAKLLGKGMKEAKEKRQEQIAKRHRLSSL

>gi|15342049|gb|AAH13296.1|AAH13296 Similar to ribosomal protein S6 [Homo sapiens]  
 MKLNI SFPATGCQKLIIEVDDECKLRTFYEKRMATEVAADALGEEWKGYVVRISGGNDKQGFPMKQGVLT  
 GRVRLLLSKGHSCYRPRRTGERKRKSVRGCIVDANLSVLNLVIVKKGEDIPLGLTDTTVPRRLGPKRASR  
 IRKLFLNLSKEDDVQYVVRKPLNKEGKKPRTAPKIQRLVTPRVLQHKRRRIALKKQRTKKNKEEAAEYA  
 KLLAKRMKEAKEKRQEQIAKRRRLSSLRASTSKSESSQK

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>gi|17474556|ref|XP\_062542.1| similar to 40S ribosomal protein S6  
(Phosphoprotein NP33) [Homo sapiens]  
MKLNISFPATGCQKLI EVDDERKLRTFYEKRMATEVAADALGEEWKGYVVRISGGNDKQGFPMKQGVLT  
HGRVRLLLSKGHSCYRPRRTGERKRKSVRGCIVDANLSVLNLVIVKKGEKDIPGLTDTTVPRRLGPKRASR  
IRKLFNLSKEDDVRQYVVRKPLNKEGKKPRTKAPKIQRLVTPRVLQHKRRRIALKKQRTKKNKEEAAEYA  
KLLAKRMKEAKEKRQEQIAKRRRLSSLRASTSKSESSQK

>gi|20552076|ref|XP\_115132.1| similar to 40S ribosomal protein S6  
(Phosphoprotein NP33) [Homo sapiens]  
MKLNIFPAAGCQKLI AVDDDEHKLRSLYEMPVATEVAADALVKHGRVLWSGSVVGTNNKPSHEAGRASRIR  
RLFNVSKEDDVFFRRRRRLNVEMLKCFKC

TABLE 1

MRB symbol	MRB name aliases	MRB RefSeq ID of GI:NA	MRB GI:AA	MRB NAME	MRB DESCRIPTION	MRB PROTEIN LENGTH	MODIFIER GI:AA
LOC120318	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	XM_061988.1	17458725	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	na	248	17737290
LOC160369	similar to ribosomal protein S6	XM_090253.1	18579097	similar to ribosomal protein S6	na	240	17737290
RPS6	phosphoprotein NP33, 40S ribosomal protein S6, ribosomal protein S6	NM_001010.2	15342049	ribosomal protein S6	RNA binding; structural constituent of ribosome; structural constituent of ribosome; structural constituent of ribosome	249	17737290

TABLE1

MRB symbol	MRB name + aliases	MRB RefSeq q_NA or GI_NA	MRB GI_AA	MRB NAME	MRB DESCRIPT ION	MRB PROT EIN LENGT H	MODIFIER GI_AA
LOC121 253	similar to 40S ribosom al protein S6 (Phosp hoprotei n NP33)	XM_06 2542.1	17474556	similar to 40S ribosomal protein S6 (Phospho protein NP33)	na	249	17737290
LOC196 391	similar to 40S ribosom al protein S6 (Phosp hoprotei n NP33)	XM_11 5132.1	20552076	similar to 40S ribosomal protein S6 (Phospho protein NP33)	na	100	

TABLE1

MRB Symbol	MRB name aliases	MRB RefSet GI_NA or GI_NA	MRB GI_AA	MRB NAME	MRB DESCRIPT ION	MRB PROT EIN LENGT H	MODIFIER GI_AA
PSMC3	TBP1,proteasome (prosome, macropain) 26S subunit, ATPase, 3	NM_002804.2 XM_006170.4	21361144	proteasome (prosome, macropain) 26S subunit, ATPase, 3	proteasome ATPase; proteasome ATPase; ATPase; transcription co- repressor; transcription co- activator	439	17738151
LOC205865	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	XM_116191.1	20533870	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	na	177	17737290
LOC137397	similar to Rim2 protein	XM_070395.1	17448285	similar to Rim2 protein	na	299	17737290